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Mechanism of lantibiotic-induced pore-formation

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Key words: nisin, lantibiotics, liposomes, protonmotive-force

Abstract

Nisin and other lantibiotics have a bacteriocidal effect against Gram-positive bacteria, and also inhibit the outgrowth of bacterial spores. The bacteriocidal effect appears to be due to the formation of pores in the bacterial membrane. In the absence of anionic membrane phospholipids, the lantibiotic nisin acts as an anion selective carrier. In the presence of anionic phospholipids, nisin forms nonselective, transient, multi-state pores in cells, proteoliposomes, liposomes and black lipid membranes. Pore formation involves distinct steps. First, nisin associates tightly with the anionic membrane surface leading to a high local concentration. This results in a disturbance of the lipid dynamics near the phospholipid polar head group-water interface, and an immobilization of lipids. In the presence of a transmembrane electrical potential above the threshold level, the molecules reorient, presumably as an aggregate, from a surface-bound into a membrane-inserted configuration. Co-insertion of bound, anionic phospholipids results in bending of the lipid surface giving rise to a wedge-like, nonspecific, water-filled pore.

Abbreviations: $\Delta\psi$ – transmembrane electrical potential, Δp – proton motive force

Introduction

Among the bacteriocins produced by bacteria, lantibiotics are the most efficient antimicrobial peptides (Klaenhammer 1993). Some lantibiotics such as Pep5 (Sahl et al. 1981, 1985), nisin and epidermin induce voltage-dependent ion current in membranes, others are enzyme inhibitors and immunologically active peptides, like ancovenin, duramycin, mersacidin and actagardine (Jung 1991). Here, we concentrate only on the pore-forming lantibiotics. The best known lantibiotic is nisin, a pore former, composed of 34 amino acids (Fig. 1). It possesses antimicrobial activity against a broad spectrum of Gram-positive bacteria (See chapter on nisin, this issue). Gram-negative bacteria, such as for instance *Escherichia coli*, are only affected by nisin when the outer membrane is disrupted (Stevens et al. 1991). Nisin has – at least – four types of antimicrobial activities, i.e. i: is capable of bacterial killing by forming pores in the cytoplasmic membrane, ii: inhibits the outgrowth of bacterial spores (Gross & Morell 1971; Morris et al. 1984). iii: might inhibit cell wall biosynthesis (Reisinger et al. 1980; Sahl 1991) and iiii: affects the activity of autolytic enzymes (Bierbaum & Sahl 1985, 1987, 1988, 1991). Concerning the second above mentioned activity the following is of interest. Studies with mutants of nisin and subtilin in which the dehydroalanine residue at position 5 has been replaced by alanine show that these mutants retain full bacteriocidal activity but are inactive in inhibiting the outgrowth of spores of *Bacillus* spp. (Liu & Hansen 1993; Chan et al. 1995a). This difference in the structure-activity relationships for the two activities clearly indicates that the mechanism of the two effects of nisin and subtilin must be different.

In relation to the best known activity of nisin, the pore formation, the following is relevant: nisin and other similar lantibiotics are cationic. Nisin is soluble in

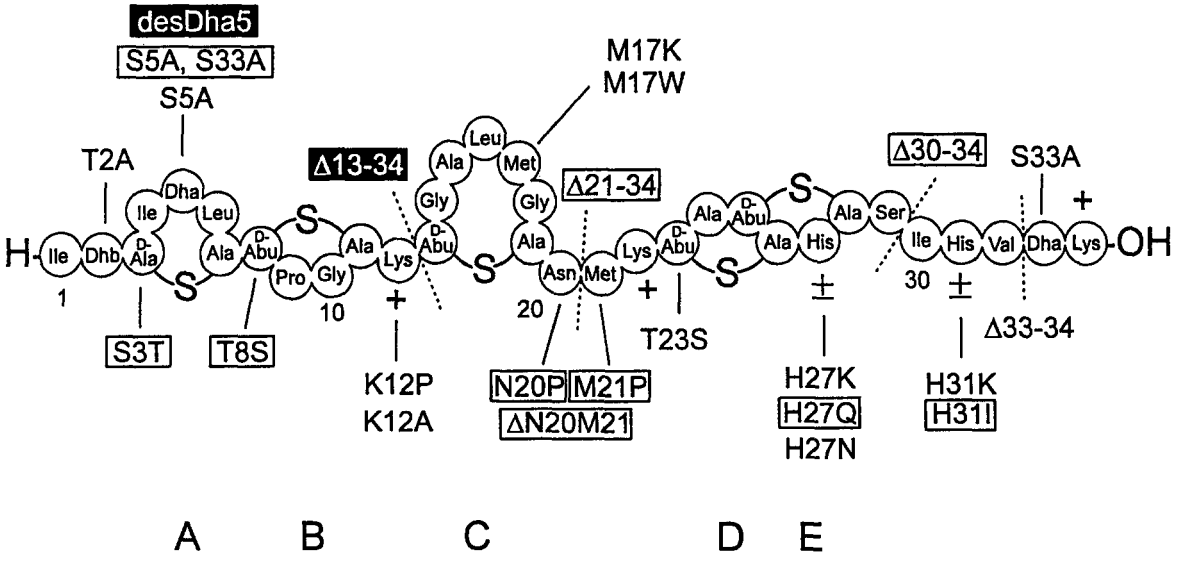

Fig. 1. Schematic structure of nisin A showing the position of modification and site-directed mutants. When indicated boxed, alteration of the molecule resulted in a dramatic loss of activity. Modifications indicated in black boxes caused an almost complete loss of antimicrobial activity. Fragmentation of the nisin molecule by proteolysis is indicated by dotted lines. Dhb, dehydrobutyrine; Dha, dehydroalanine; D-Ala$_s$, alanine moiety of lanthionine or 3-methyllanthionine; D-Abu, α-aminobutyric acid moiety of 3-methyllanthionine.

aqueous solution at pH 2, but at high pH values it forms oligomers and is inactivated (Liu & Hansen 1990). Pore formation by nisin in the cytoplasmic target membrane results in the efflux of ions, amino acids and ATP and a collapse of the protonmotive-force (Δp) (Bruno et al. 1992; Gao et al. 1991, García Garcerá et al. 1993; Okereke & Montville 1992; Ruhr & Sahl 1985; Sahl 1991, Sahl et al. 1995). Nisin requires a trans-negative electrical potential ($\Delta\psi$) for its pore forming action (Sahl et al. 1987; Sahl 1991; Kordel & Sahl 1986). Nisin displays also activities on liposomes. It forms pores in anionic phospholipid containing liposomes and has anion carrier activity in liposomes which do not contain these lipids. Since liposomes can be used to study pore formation by nisin, it seems that *in vivo* no binding to a putative target cell *protein*-receptor is necessary. On the other hand the possibility remains, that anionic phospholipids act as "nisin receptors" and that binding to these is required for pore formation. Here we summarize a large variety of work on the action of cationic lantibiotics on intact cells, proteoliposomes, liposomes and black lipid membranes, converging to the current model(s) for the molecular mechanism(s) of pore formation by nisin and possibly also that by other lantibiotics.

Anion-carrier activity

Although the primary antimicrobial activity of nisin is supposed to be associated with its pore forming activity it is of interest that nisin and epilancin K7 (Driessen et al. 1995) induce the release of the fluorophore carboxyfluorescein from phosphatidylcholine liposomes. The presence of anionic phospholipids in the liposomes strongly inhibits this activity (García Garcerá et al. 1993). The ratio of the number of molecules nisin/liposomes determines the speed with which carboxyfluoresceine is released by the liposomes; saturation occurs at approximately 100 molecules nisin/liposome. No threshold $\Delta\psi$ (transmembrane electrical potential) is necessary for the nisin carrier activity, but a $\Delta\psi$ inside negative enhances (García Garcerá et al. 1993), and a $\Delta\psi$ inside positive inhibits (Driessen et al. 1995). The nisin induced anion flux is slow and does not result in a major dissipation of the $\Delta\psi$. In the absence of stable interactions with anionic phospholipids, the cationic nisin appears to act as an anion-carrier and functions according to the following mechanism. When added from the outside, nisin binds to the lipid surface and transverses the membrane in a $\Delta\psi$ (inside negative) stimulated manner. It then binds the anion (such as carboxyfluorescein) on the inside, and subsequently transverses

the membrane as a binary complex with the anion. On the outer surface, the anion is released and nisin recycles to bind another anion on the inside. Penetration of the lantibiotic into the membrane is likely to be rate-limiting, and for epilancin K7 direct evidence has been obtained, that this step is promoted by $\Delta\psi$ (Driessen et al. 1995). Why do anionic phospholipids inhibit the carrier activity of nisin? Nisin may remain bound to the negative surface charge of anionic phospholipid as a partially neutralized complex at the lipid surface or interface. The "sites" on nisin that might bind anions in phosphatidylcholine liposomes may be already occupied by the anionic lipids and would thus not be available to bind other negatively charged groups. Specific interaction of lantibiotics, nisin and epilancin K7, with negatively charged phospholipids has been well documented (Driessen et al. 1995). In the case of phosphatidylglycerol membranes no carrier activity at all is measured. Since target bacterial membranes are rich in anionic phospholipid, low anion carrier activity is to be expected *in vivo*.

Lipid binding

Fluorescence studies show that nisin tightly associates with the anionic surface of phosphatidylglycerol liposomes but not (even in the presence of a $\Delta\psi$) with the surface of phosphatidylcholine liposomes (Driessen et al. 1995). NMR and fluorescence studies show that nisin disturbs the lipid bilayer structure, presumably dehydrating the phosphatidylglycerol headgroups. Nisin seems to immobilize some of the phosphatidylglycerol, and restricts the fluidity of phosphatidyl glycerol membranes near the interface between the aqueous phase and the lipid head group (Driessen et al. 1995). Furthermore, nisin causes aggregation of phosphatidylglycerol liposomes and promotes the transfer between these liposomes of octadecylrhodamine B. Further studies with a novel cationic lantibiotic, epilancin K7, indicate that this molecule, or at least the tyrosine residue located in the middle of the molecule, penetrates into the hydrophobic carbon region of the lipid bilayer upon the imposition of a $\Delta\psi$. NMR data indicate that nisin in solution adopts a rather flexible structure with an amphiphilic character (Van de Ven et al. 1991). Nisin binds to micelles of zwitterionic dodecylphosphocholine or of anionic sodiumdodecylsulphate (Van den Hooven et al. 1993; Lian et al. 1991, 1992) in approximately similar conformations with the charged side exposed to the aqueous phase (See chapter on 3D-structure, this issue).

Energy requirement for pore-formation

It has to be stressed that a sharp distinction exists between the pore forming activity and the anion carrier activity. Nisin pores cause a rapid (contrarily to the carrier activity) dissipation of $\Delta\psi$ (Ruhr and Sahl 1985; Gao et al. 1991; Okereke and Montville 1992) and ΔpH (Gao et al. 1991) in intact cells, cytoplasmic membrane vesicles and liposomes. Nisin induced pores have a low specificity (Sahl 1991), while the carrier activity is anion selective. The carrier activity is found in pure phosphatidylcholine liposomes, but not in pure phosphatidylglycerol liposomes. On the other hand nisin dissipates the Δp in phosphatidylglycerol liposomes but not in pure phosphatidylcholine liposomes. Pore formation strictly requires a $\Delta\psi$, whereas the carrier activity does not need a $\Delta\psi$. Nevertheless, one study (Gao et al. 1991) demonstrated $\Delta\psi$ dissipation in *E. coli* liposomes without the need of a threshold level. However, the concentrations used in that study are 15–50 fold higher than in a study in which treshold values were shown to be necessary.

Studies with liposomes show that the lipid composition and the magnitude of the $\Delta\psi$ above the treshold affect the action of nisin. (Abee et al. 1991). Nisin also dissipates ΔpH, trans-alkaline, in liposomes. Dissipation of the ΔpH displays the same concentration dependence as dissipation of $\Delta\psi$, suggesting that ΔpH is as efficient as $\Delta\psi$ in promoting pore formation. Nisin also dissipates Δp in proteoliposomes, in which cytochrome c oxidase has been incorporated as a H^+ pump. Higher concentrations of nisin are needed for the Δp dissipation as compared to liposomes. To all likelihood this is due to the pumping activity of the cytochrome c oxidase. Threshold values between 50 and 100 mV were measured when intact cells were treated with Pep5, nisin, subtilin, epidermin and gallidermin. All these peptides cause rapid efflux of K^+, amino acids and ATP from the cytoplasm of various Gram-positive test bacteria and concomitantly $\Delta\psi$ decreases. Efflux and depolarization are much faster with energized cells, than with starved cells. In addition, cells that have been de-energized with uncouplers of the oxidative phosphorylation are completely insensitive.

At a low $\Delta\psi$ within the range of 10 to 40 mV the lantibiotics nisin, Pep5, subtilin, gallidermin and

epidermin do not induce any permeability in artificial membranes. At threshold values between 50 and 80 mV, negative at the trans side, nisin and Pep5 cause an increase in permeability of several orders of magnitude. The membrane conductance is an exponential function of the voltage applied. When the trans side is positive, no conductance increase is brought about neither by nisin nor by Pep5. This is in agreement with the observations that also in whole cells and liposomes, a threshold potential, inside negative, is needed for nisin pore formation. Threshold values may reflect the energetically rate limiting step of the pore formation, that is the insertion of the peptide(s) into the membrane. Gallidermin and epidermin, on the other hand increase the conductivity irrespective of the polarity of the applied voltage. Single channel measurements suggest that the lantibiotics act by the formation of transient channels with highly variable single-channel conductances. The channels lifetime is in the order of milliseconds to seconds. Pores that have been formed remain stable for a short period of time even when the electrical potential across the black lipid bilayer is lowered below the threshold value of pore-formation. The channels diameter is estimated to be around 1 nm for low (100–500 nM) concentrations of nisin and Pep5, and around 2 nm for subtilin (Benz et al. 1991).

How are pores formed: A hypothetical model

The small size of the 34 residue peptide nisin and of the other lantibiotics excludes the possibility that one molecule could span a membrane several times, which would be needed for the formation of a channel from one single molecule. Therefore one has to assume that several molecules participate in channel formation, forming for instance a "barrel-stave-pore". Artificial membranes also give some information on the manner of channel formation. Single channel measurements do not reveal distinct steps but a variety of fast current fluctuations on the millisecond scale, including bursts and pulse like spikes. At higher voltages both the lifetime and the fluctuations increase (Benz et al. 1991). Apparently no stable regular channels are formed like those known for instance for gramicidin. This holds true for nisin, Pep5 and subtilin, and might be explained by the formation of oligomers with varying numbers of participating molecules, leading to multi-state channels and varying conductance levels. In contrast epidermin and gallidermin have a lower threshold and a longer channel lifetime.

Several models have been proposed to explain the pore-forming ability of nisin. The "insertion model" assumes that the molecules are initially bound at the lipid surface, and subsequently in the presence of a $\Delta\psi$, they may flip into a membrane-spanning orientation. The membrane-inserted molecules may form a cluster around a central pore as in the above mentioned "barrel-stave model". To all likelihood the order of events will be first insertion and subsequently aggregation. Alternatively, a "wedge model" (Fig. 2) can be envisioned where the amphiphilic molecules adhere to the surface of the membrane causing destabilisation of the bilayer structure thereby promoting pore formation, such as proposed for annexins (Demange et al. 1994). Binding of nisin to anionic phospholipids indeed results in a local disorder of the phospholipid headgroups. This process is, however, not directly responsible for pore formation as there is no general loss of the permeability barrier or integrity of the membrane. Pore-formation requires the presence of a high $\Delta\psi$. Nisin has a strong effect on the lipid dynamics, (dehydration of the lipid surface, non-bilayer intermediate structures, stimulation of lipid transfer between liposomes, i.e. bilayer-bilayer contacts) when incubated with liposomes at high concentrations. The PG surface-bound form of nisin may represent a true intermediate in the pore formation process. $\Delta\psi$ may change the orientation of nisin in the membrane relative to the plane of the membrane, forming a wedge (Fig. 2). By carrying the anionic lipids along it may induce a local bending of the membrane surface. In effect this will result in the formation of a metastable non-selective water-filled pore when sufficient nisin molecules cooperate in bending and locally disturbing bilayer structure. As suggested by the NMR data, nisin may expose its charged side to the aqueous phase under these conditions. An important aspect of this model is that the actual pore is formed by an array of nisin molecules that temporarily force the lipids in a thermodynamically unfavourable non-bilayer conformation. Such pores are intrinsically instable, as the lipid will try to rearrange into a bilayer structure. Moreover, since the association and dissociation of nisin oligomers is likely to be a dynamic event, lowering of the magnitude of $\Delta\psi$ below the threshold of insertion, will result in a relaxation of the nisin molecules to the surface-bound state and a disassembly of the pore.

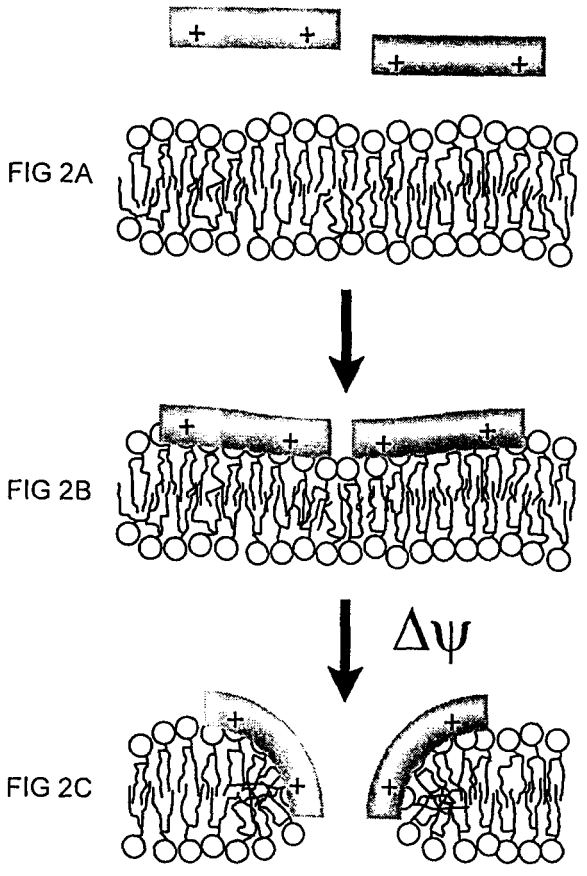

Fig. 2. Wedge-like model of nisin induced pore-formation. At first nisin is in solution (Fig. 2A) Then nisin binds to the anionic membrane surface leading to a high local concentration and disturbance of the lipid dynamics (Fig. 2B). In the presence of a $\Delta\psi$ above the threshold level, the molecules insert into the membrane. Co-insertion of bound, anionic phospholipids results in bending of the lipid surface giving rise to a wedge-like, nonspecific pore (Fig. 2C).

Nisin fragments and site-directed mutants

How do the site directed mutagenesis studies and results of engineering of the nisin molecule (See chapter on engineering, this issue) (Summarized in Fig. 1) contribute to pore-formation-models? NMR studies by Lian et al. (1991, 1992) indicated flexibility of the "hinge" between rings A and B and between ring C and D. In solution the flexibility of this first hinge (B/C) was found nearly as high as the second hinge (C/D). Also other NMR studies indicated a highly flexible hinge region in nisin connecting rings ABC with DE (Van den Hooven et al. 1993; Van de Ven et al. 1991). According to the wedge-model of pore-formation, this region may act as a twist that allows nisin to bend the lipid surface (Driessen et al. 1995). Attempts to immobilize these rings by either deleting residues 20 and 21, or by replacing them by proline residues, indicate a dramatic loss of activity. An intact ring A is important for activity. This is shown, for example, by the activities of the two major breakdown products of nisin, formed by acid-catalysed hydrolysis of the two dehydroalanine residues.

Nisin$^{1-32}$, lacking the two C-terminal residues, has full bacteriocidal activity, but (*des*-ΔAla$_5$)-nisin$^{1-32}$, in which dehydroalanine 5 is also missing, is inactive (Chan et al. 1989). The observation that the ΔAla$_5$-Ala mutant retains full activity (Chan et al. 1995a) suggests that it is the opening of the ring rather than the absence of the dehydro residue which leads to the loss of activity in (*des*-ΔAla$_5$)nisin$^{1-32}$. Indeed, even a distortion of ring A produced by converting D-Ala$_s$ to D-Abu at position 3 (achieved by a serine to threonine substitution in the structural gene) results in a complete loss of activity. The integrity of ring C is similarly essential for activity: proteolytic cleavage of either the Ala$_{15}$-Leu$_{16}$ bond (Chan et al. 1993, 1995b) or of the Lys$_{17}$-Gly$_{18}$ bond in a Met$_{17}$ to Lys mutant leads to complete loss of activity. It remains to be established whether the additional charges introduced by proteolysis contribute to this loss of activity. Studies of other proteolytic fragments of the molecule have identified other residues which are important for bacteriocidal activity. For exemple, while nisin 1-32 has full activity, the further removal of Ile$_{30}$-His$_{31}$-Val$_{32}$ to give nisin 1-29 leads to a 30 fold drop in activity (Chan et al. 1993, 1995b). The fragment nisin$^{1-12}$ is a particularly interesting one since, while it is itself inactive, it antagonizes the bacteriocidal effects of nisin (Chan et al. 1995b) and the nisin-induced $\Delta\psi$ dissipation in cytochrome c oxidase proteoliposomes (J. Clark & G. Moll, unpublished work). This suggests that rings A, B and C are required for binding, but that more C-terminal portions of the molecule are required in addition for pore formation. It remains to be established whether the rings A, B and C are involved in the oligomerisation of nisin and/or its binding to phospholipids in the membrane.

According to the wedge model, an electrostatic interaction between the cation side-chains of nisin and anionic lipids is responsible for tight surface binding. Mutagenesis studies demonstrate that the positive charge on position 12 is not essential for the antimicrobial activity. In contrast, for optimal activity the imidazole groups of histidine at position 27 and 31 need to be protonated. Nisin Z is a natural occurring variant

of nisin A with the histidine at position 27 replaced by an asparagine. Replacement of these residues by lysine has little effect on the activity but improves the solubility of nisin. Lysine residue 34 is not essential as truncation of the last two residues at the carboxy-terminus has little effect on the activity. The role of the lysine residue at position 22 has not been investigated sofar.

Although there is no direct evidence supporting this view at this stage, it seems conceivable that rings ABC or parts thereof are involved in oligomerization of nisin, while the positively charged carboxy-terminal moiety of the molecule inserts in the membrane as depicted in Fig. 2. Further studies on the mode of action of nisin, nisin-derivatives and site-directed mutants will reveal the intimate features of pore-formation.

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